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14. ABSTRACT

TMPRSS2-ERG fusion transcripts have been shown to be expressed in a majority of prostate cancer (PC) patients due to chromosomal translocations or deletions involving the TMPRSS2 gene promoter and the ERG gene coding sequence. These alterations cause androgen dependent ERG transcription factor expression in PC patients. We and others have shown that chemokine receptor CXCR4 expression is upregulated in PC tumor cells and its ligand, CXCL12, is expressed in bone stromal cells. The CXCL12/CXCR4 axis transactivates EGFR family members in in PC cells and functions in PC progression to enhance invasion and metastasis. To address the mechanism of CXCL12/CXCR4 transactivation of EGFR family members, we evaluated the location of transactivation at cellular, cell surface and lipid raft microdomains. To determine the impact of CXCL12/CXCR4 activation on initial colonization of bone tissue, we targeted CXCL12/CXCR4 axis with a small molecule CXCR4 inhibitor AMD3100. Results of the current study show that (a) CXCL12/CXCR4 transactivation of EGFR members is confined to lipid raft membrane microdomains and (b) targeting CXCL12/CXCR4 axis with AMD3100 resulted in delayed growth of bone tumors. These findings demonstrate CXCL12/CXCR4 transactivation of EGFR members in lipid raft membrane microdomains contribute to initial colonization and growth of PC cells to bone metastatic site.

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INTRODUCTION:

Specific chromosomal alterations were recently discovered in prostate cancer (PC) patients using bioinformatics analysis of micro-array data (1, 2). Prevalent chromosomal alterations due to interstitial deletion or translocations result in the fusion of the androgen responsive TMPRSS2 gene promoter with Ets transcription factor coding sequences. Ets family of transcription factors ERG, Etv1 and Etv4, were shown to be involved in chromosomal alterations. Among these transcription factors, ERG was identified as being commonly fused with the TMPRSS2 gene in a majority of PC patients (1-3). Some reports suggest that the presence of fusions is associated with a poor outcome (4-6) and that specific ERG isoform expression correlates with aggressive disease characteristics (7). Other studies suggest that these chromosomal alterations alone are not associated with patient outcome, but that copy number increase of the alterations results in poor outcomes (8). Previous reports demonstrate that prostate specific overexpression of the ERG gene in transgenic mouse models results in the development of prostate intraepithelial neoplasia (PIN) without progression to carcinoma (9, 10). TMPRSS2-ERG translocations have also been identified in the low grade PIN lesions adjacent to cancer suggesting that ERG expression contributes to PIN development (11). Further, two recent reports demonstrate that ERG overexpression alone is not sufficient for prostate cancer progression; additional loss of PTEN co-operates in the development of highly invasive prostate adenocarcinoma (12, 13). Studies with patient tumor tissues confirmed *in vivo* findings that alterations in ERG and PTEN genes in prostate cancer patients results in the development of aggressive disease (14). The molecular targets related to androgen mediated activation of TMPRSS2-ERG are currently unknown; herein, we provide evidence that chemokine receptor CXCR4 is one such target of androgens in PC cells.

CXCR4 is a chemokine receptor that has been shown to function as a key receptor for homing of circulating tumor cells to secondary sites; its ligand CXCL12 is highly expressed at these metastatic sites (15-17). CXCL12/CXCR4 signaling has been shown to be involved in the adhesion, migration, invasion, and metastasis of PC cells in laboratory model systems (16, 17). We have recently shown that CXCL12/CXCR4 signaling transactivates members of the Epidermal Growth Factor Receptor (EGFR) family in membrane microdomains of prostate cancer cells, and this transactivation contributes to the expansion of intraosseous metastatic deposits (18). CXCR4 has been shown to be deregulated in tumor cells through transcriptional mechanisms. Prostate tumors and metastases express higher levels of CXCR4 compared to non tumor tissue (19-21) and this overexpression is associated with aggressive disease in patients (20, 22).

BODY:

Specific Aim 1: To test hypothesis that the ERG transcription factor promotes PC progression via regulation of CXCR4 expression and that this process is driven by androgens.

Reported in previous two years.

Specific aim 2: To test the hypothesis that CXCR4 activation promotes PC progression via transactivation of certain EGFR family members:

2.1 To test the hypothesis that CXCR4 signaling transactivates specific EGFR family members.

CXCL12/CXCR4 axis activates HER2 and EGFR in lipid raft membrane microdomains: Western blot analysis of biotinylated cell lysates, biotinylated membrane microdomains and total cell lysates for EGFR total and phosphor members (16-22 months)

Identify the individual EGFR family members transactivated by CXCL12/CXCR4: To determine the cellular location of CXCL12/CXCR4 transactivation of EGFR family members, we treated PC-3 and C4-2B cells with CXCL12 and performed Western blot analysis for HER2, pHER2, EGFR and pEGFR. CXCL12 did not significantly altered total cellular HER2 or EGFR phosphorylation in in PC cells. Then, we analyzed HER2 and EGFR phosphorylation in cell surface populations with biotinylation methodology. CXCL12 did not significantly change HER and EGFR phosphorylation in cell surface expressed receptors. We analyzed HER2 and EGFR phosphorylation in lipid raft microdomains and cytosol and membrane fractions, CXCL12 induced both HER2 and EGFR phosphorylation in lipid raft membrane microdomains in PC-3 cells. Thus, CXCL12/CXCR4 axis in lipid raft membrane microdomains transactivate EGFR and HER2 in prostate cancer cells.

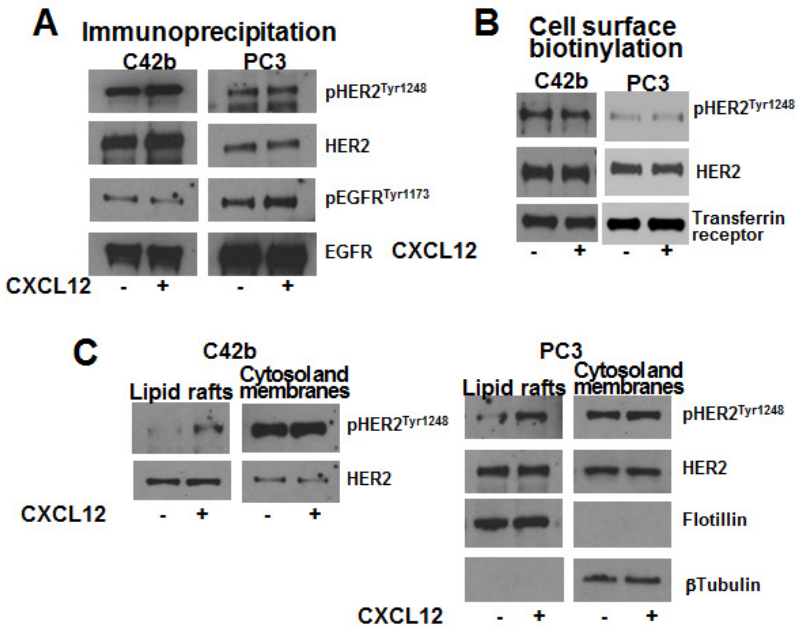

Figure 1: CXCL12/CXCR4 transactivates HER2 and EGFR in lipid raft membrane microdomains: Panel A. C4-2b and PC-3 cells were untreated or treated with CXCL12 at 200ng/ml for 15 min and total cellular lysates were immunoprecipitated with anti-HER2 and anti-EGFR antibodies and immunoblottd with $pHER2^{Tyr1248}$, HER2, $pEGFR^{Tyr1173}$ and EGFR. Panel B. C4-2b and PC-3 cells were untreated or treated with CXCL12 at 200ng/ml for 15 min. Cells were biotinylated and cell extracts were affinity captured with anti-streptavidin beads and immunobolted with $pHER2^{Tyr1248}$, HER2 and cell surface protein marker transferrin receptor. Panel C. C4-2b and PC-3 cells were untreated or treated with CXCL12 at 200ng/ml for 15 min and lipid raft microdomains and cytosol and membrane fractions were isolated. Both fractions were immune blotted with $pHER2^{Tyr1248}$ and HER2. In PC-3 cells both fractions were immunobloted with lipid raft marker Flotillin and cytosol marker beta Tubulin.

Determine the role of individual EGFR family members in CXCL12/CXCR4 mediated proMMP-9 secretion and invasion of PC-3 cells: siRNA transfection of EGFR family members and transient transfection studies (20-24 months).

We transfected four different siRNA plasmids for EGFR in PC-3 cells and transfections resulted in downregulation of EGFR in PC-3 cells. Gelatin zymography experiment is in progress to determine the impact of EGFR knockdown on MMP9 expression.

2.2 Intratibial model to target the CXCR4 and EGFRHER2 functions PC cells.

CXCR4 targeting delays intratibial tumor growth in PC-3 cells: We implanted PC-3 cells expressing luciferase construct and treated mice with CXCR4 inhibitor AMD3100 with a subcutaneous pump system. Tumor growth was monitored with luciferase imaging. AMD3100 treatment significantly reduced the tumor growth compared to control mice as measured by luciferase signal. X-Ray analysis of bone tumors showed osteolysis of bone tumors in untreated mice and AMD3100 treated mice show no signs of osteolysis. CXCR4 inihibitor significantly inhibited tumor growth and bone tumor osteolysis in intratibial bone tumor growth model.

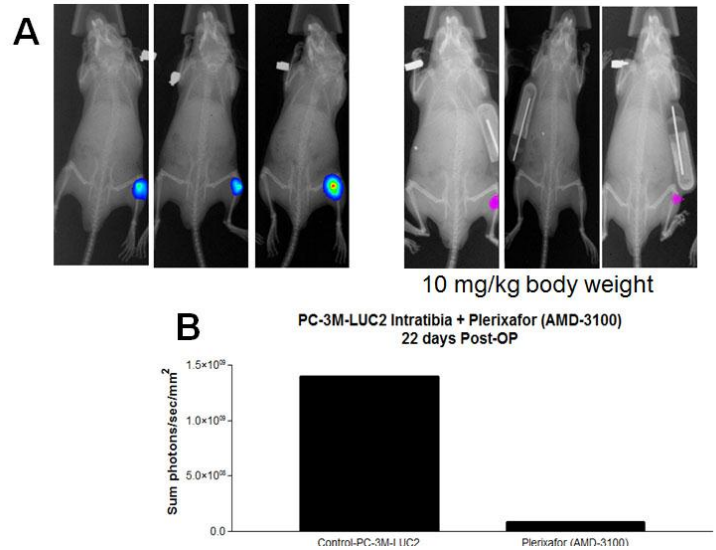

Figure 2. AMD3100 inhibits intratibial tumor growth of PC-3 cells.PC-3ML_Luc cells were implanted in tibiae of mice and treated with AMD3100. Panel A shows a representative 3 mice images of X-rays and luciferase imaging. Panel B is sum of photons which represents tumor burden in animals.

KEY RESEARCH ACCOMPLISHMENTS:

1. CXCL12/CXCR4 trans activated HER2 and EGFR receptors in PC cells and lipid raft microdomains is the site for transactivation.
2. Targeting CXCL12/CXCR4 axis with AMD3100 inhibits PC bone tumor growth.

REPORTABLE OUTCOMES:

Podium Presentation:

Role of ERG and CXCR4 in Prostate Cancer Bone Metastasis. 7th Annual National Symposium on Prostate Cancer. Clark Atlanta University, Atlanta, GA. May 23-24, 2011.

Publication: Review article

St. John, J*, Powell, K*, Conley-LaComb, M, and **Chinni, S.R.** *TMPRSS2-ERG* fusion gene expression in prostate tumor cells and its clinical and biological significance in prostate cancer progression. *Journal of Cancer Science and Therapy* (In Press).

CONCLUSION:

Our results suggest that CXCL12/CXCR4 axis activates EGFR and HER2 members of EGFR family. Biochemical studies demonstrate that this transactivation is exclusively occurring in lipid raft membrane microdomains in PC cells. Targeting CXCR4 receptor with small molecule inhibitor AMD3100 show that tumor growth in bone site is inhibited. This inhibition of bone tumor growth is accompanied by reduced tumor mediated osteolysis at bone site. These studies identify that GPCR mediated activation of growth family factor receptors particularly CXCR4

transactivation of HER2 and EGFR is confined to lipid raft microdomains in PC cells and targeting the receptor activity inhibits bone tumor growth in intratibial animal model.

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APPENDICES

ROLE OF ERG AND CXCR4 IN PROSTATE CANCER PROGRESSION.

Chinni, SR. Singareddy R, and St. John J

TMPRSS2-ERG fusion transcripts have been shown to be expressed in a majority of Prostate Cancer (PC) patients due to chromosomal translocations or deletions between the TMPRSS2 gene promoter and the ERG gene coding sequence. The TMPRSS2 promoter contains androgen receptor binding sites, and these alterations cause androgen dependent expression of ERG transcription factor in PC patients. Overexpression of ERG factor confers growth and invasive advantage to PC cells.

We and others have shown that chemokine receptor CXCR4 expression is upregulated in PC tumor cells and its ligand CXCL12 is highly expressed in bone stromal cells. The CXCL12/CXCR4 axis functions in PC progression to enhance cell invasiveness and metastatic growth. Towards this end, we recently identified CXCL12/CXCR4 transactivation of the epidermal growth factor receptor system to be an upstream signaling pathway for PC cell invasion and metastatic growth at secondary sites. To address the regulation of CXCR4 expression, we identified several putative ERG consensus binding sites in the promoter region of CXCR4. We hypothesized that androgen dependent regulation of the ERG transcription factor could induce CXCR4 expression in PC cells. Subsequent CXCR4 localization to raft membrane microdomain and signaling contributes to PC cell invasion and metastasis.

Using a variety of methods including RT-PCR, chromatin immunoprecipitation, Western Blot Analysis, siRNA transfection, and chemoinvasion assay, we show that (a) prostate tumor cells co-express higher ERG and CXCR4 compared to benign tissue; (b) CXCR4 expression is increased in the TMPRSS2-ERG fusion positive cell line; (c) ERG transcription factor binds to the CXCR4 gene promoter; (d) synthetic androgen (R1881) upregulates both ERG and CXCR4 in TMPRSS2-ERG fusion positive VCaP cells; (e) siRNA mediated downregulation of ERG resulted in a loss of androgen dependent regulation of CXCR4 expression in VCaP cells; (f) R1881 activated TMPRSS2-ERG expression functionally activates CXCR4 in VCaP cells; (g) CXCR4 activation in raft membrane microdomains leads to Src and EGFR family member activation. These findings identify CXCR4 as a target for androgen activated TMPRSS2-ERG fusions in PC cells. Subsequent CXCR4 function in raft membrane microdomains confers invasive and metastasis phenotype to cancer cells. Together these results may provide a link between TMPRSS2-ERG translocations and enhanced metastasis of tumor cells via CXCR4 function in PC cells.

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Review Article Open Access

TMPRSS2-ERG Fusion Gene Expression in Prostate Tumor Cells and Its Clinical and Biological Significance in Prostate Cancer Progression

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Abstract

TMPRSS2-Ets gene fusions were identified in prostate cancers where the promoter of transmembrane protease, serine 2 (TMPRSS2) fused with coding sequence of the erythroblastosis virus E26 (*Ets*) gene family members. *TMPRSS2* is an androgen responsive transmembrane serine protease. *Ets* family members are oncogenic transcription factors that contain a highly conserved *Ets* DNA binding domain and an N-terminal regulatory domain.

Fusion of these gene results in androgen dependent transcription of *Ets* factor in prostate tumor cells. The *ERG* is the most common fusion partner with *TMPRSS2* promoter in prostate cancer patients. The high prevalence of these gene fusions, in particular *TMPRSS2-ERG*, makes them attractive as potential diagnostic and prognostic indicators, as well as making them a potential target for tailored therapies.

This review focuses on the clinical and biological significance of *TMPRSS2-ERG* fusions and their role in PC development and progression.

Keywords: TMPRSS2-ERG; Prostate Cancer; Clinical; Biological significance

Introduction

Prostate cancer (PC) is the most common form of cancer found in American men and the second leading cause of cancer death. The American Cancer Society [1] estimates that in 2012 there were 241,740 newly diagnosed cases of PC, with an estimated 28,170 men dying as a result of the disease. This means that approximately 28.5% of cancers and 3.5% of cancer related deaths in men are due to PC. Because of research that has generated improved treatments and earlier diagnosis, the five-year survival rate has significantly increased in PC patients [1]. However, the key molecular mechanisms responsible for the initiation and progression of PC remain largely unknown.

Gene fusions were known to be prevalent in liquid tumors, such as *BCR-ABL* in chronic myelogenous leukemia, but had not been identified in solid epithelial tumors. The first PC fusion genes, involving the promoter of transmembrane protease, serine 2 (*TMPRSS2*) fused with coding sequence of the erythroblastosis virus E26 (*Ets*) gene family members, were identified in 2005 by Tomlins et al. [2]. *TMPRSS2* is a prostate specific, androgen responsive, transmembrane serine protease. *Ets* family members are oncogenic transcription factors that contain a highly conserved *Ets* DNA binding region and an N-terminal regulatory domain. The *ETS* domain serves as a DNA binding recognition site, as well as a protein-protein interaction site commonly used for interactions with other transcription factors [3-5]. Therefore, the fusion of these genes leads to the production of *Ets* transcription factors under the control of the androgen sensitive promoter elements of *TMPRSS2*. This allows for a situation in which androgen-bound androgen receptor can bind these regions of *TMPRSS2*, resulting in the overexpression of *Ets* gene family members; these *Ets* members can then induce their target gene expression (Figure 1) [2]. The most common of these fusions is with *ERG* (*Ets* related gene), a member of the *Ets* family, resulting in the *TMPRSS2-ERG* fusion. The *TMPRSS2-ERG* fusion has been identified in approximately 50% of PC cases. *TMPRSS2* has also been identified in fusions with *Ets* family members *ETV1, ETV4,* and *ETV5* in PC. The prevalence of these gene fusions, in particular *TMPRSS2-ERG*, makes them attractive as potential diagnostic and prognostic indicators, as well as making them potential targets for tailored therapies.

Due to its prevalence in PC, the primary focus of this review will be on the *TMPRSS2-ERG* fusion regarding its clinical significance, biological role in PC development, and progression. This review will therefore discuss the different forms of the *TMPRSS2-ERG* fusion found in PC patients, as well as the clinical associations found between fusion positive PCs and patient outcome and disease aggressiveness will be examined. Additionally, *in vivo*, *in vitro*, and gene expression studies will be evaluated to consider the biological role of the *TMPRSS2-ERG* fusion.

TMPRSS2-ERG Fusion Gene Express Alternatively Spliced Transcript Variants in Prostate Cancer

The first two fusion genes found in PC, *TMPRSS2-ERG* and *TMPRSS2-ETV1*, were discovered in 2005 by Tomlins et al. [2]. Shortly thereafter, *TMPRSS2-ETV4* was identified [6]. Other fusions involving *Ets* family members found in PC include: *HERV_K_22q11.23-ETV1, SLC45A3-ETV1, C15orf21-ETV1, HNRPA2B1-ETV1* [7], *KLK2-ETV4, CANT1-ETV4* [8], *TMPRSS2-ETV5, SLC45A3-ETV5* [9], *SLC45A3-ERG, DDX5-ETV4, FLJ35294-ETV1* [10], and *NDRG1-ERG* [11]

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(Figure 2). These rearrangements and translocations can occur due to an intrachromosomal deletion, such as with *TMPRSS2-ERG*, as well as from an interchromosomal translocation, such as with *TMPRSS2-ETV4*. In the case of *TMPRSS2-ERG,* the rearrangement occurs either by a ~3 Mb interstitial deletion on a single copy of chromosome 21, or by a chromosomal translocation. The high prevalence of *TMPRSS2-ERG* fusions suggests that this region is a hot spot for chromosomal rearrangements in PC. Prostate cancer targeted exome sequence studies also confirm the expression of fusion genes in PC patient tissue [12]. The other 5' fusion partners of *Ets* family members are only present in a small number of PC cases and tend to be located on different chromosomes.

Wild-type *ERG* has been shown to exist as multiple different mRNA transcript variants due to alternative splicing. This alternative splicing of native *ERG* mRNA transcripts results in the expression of different isoforms of the *ERG* protein [3,13]. In accordance with this, many different splice variants of the *TMPRSS2-ERG* fusion have been identified. The differences between these variants can be found in the exons included in the transcript of that particular variant. These include T1-E4 [2], T1-E2, T4-E4, T4-E5, T5-E4 [14], T1-E5 [15], T1-E3 [16], T3-E4, T2-E2, T1-E3,5, T1-E2,3,4,6, T2-E4, T1-E6, T1-E3a4, T1-E3b4, T1-E3c4 [17], T1-E6,4 [18], T2-E5 [19] (T represents the last exon of *TMPRSS2* in the fusion; E represents the first *ERG* exon included, as depicted in Figure 3). Among the *TMPRSS2-ERG* fusion transcript variants, T1-E4 is the most common. Additionally, a variably expressed 72-bp exon has been identified that can be expressed in several of the *TMPRSS2-ERG* isoforms [13].

The *TMPRSS2-ERG* fusion proteins are most commonly expressed as N-terminal truncated *ERG* proteins, due to the loss of the 5' *ERG* native exons. The 5' *TMPRSS2* exons included in the fusion transcripts are generally non-coding and are usually not translated into protein product. However, there are several transcript variants that result in both *TMPRSS2* and *ERG* exons being translated into protein. For example, fusion protein transcript variant T2-E4 is expressed as a true fusion protein product with part of *TMPRSS2* exon 2 being expressed in the N-terminus of the fusion protein fused to N-terminal truncated *ERG* [16,17]. The DNA binding *ETS* domain and transactivation domain of the *ERG* protein are located in the C-terminus region of the protein [3]. Therefore, it is likely that, despite the N-terminal truncation, the biological functions of DNA binding and protein-protein interactions via the *ETS* domain remain intact in *TMPRSS2-ERG* fusion proteins. Due to this, it is possible that the different *ERG* fusion isoforms have similar biological functions as their wild-type *ERG* counterparts, and mediate PC progression due to overexpression. Whether novel biological functions of the *ERG* fusion isoforms lend to their oncogenic phenotype remains to be further elucidated.

Figure 2: Additional *Ets* fusions identified in prostate cancer. Several *Ets* factors have been identified in fusion genes in prostate cancer, including *ERG*, *ETV1*, *ETV4*, and *ETV5*. In addition to *TMPRSS2*, numerous other 5' fusion partners of these *Ets* family members have been identified.

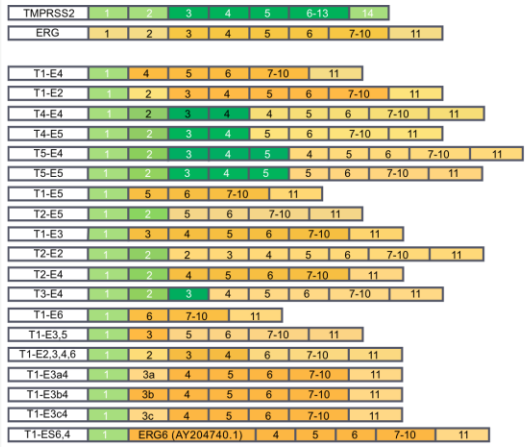

Figure 3: *TMPRSS2-ERG* fusion variants. Numerous variations of the *TMPRSS2-ERG* fusion have been identified, with the most common being T1-E4. As shown, these variants differ in the exons from *TMPRSS2* and *ERG* found in the fusion. T2-S6,4 is a variant in which the fusion is formed between exon 2 of *TMPRSS2* and 95 nucleotides that were shown to be identical to a portion of *ERG* splice form 6, which is then followed by *ERG* exon 4. The native transcripts of *TMPRSS2* and *ERG* are shown on top for reference, with *TMPRSS2* in green and *ERG* in gold. Light shading represents untranslated regions; dark shading represents open reading frame. T indicates the last exon of *TMPRSS2* in the fusion; E indicates the first *ERG* exon included.

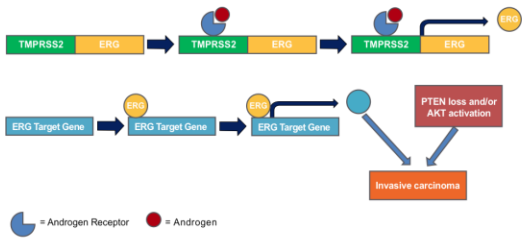

Figure 1: Biology of *TMPRSS2-ERG* gene fusions in prostate cancer. Association of bound androgen receptor with *TMPRSS2* in fusion genes results in the upregulation of *ERG* transcription (or other *Ets* gene family members, i.e. *ETV1*, *ETV4*, and *ETV5*). This over production of *ERG* can then exert its effects by binding target gene promoter regions, which results in their activation or inhibition, and the generation of a neoplastic phenotype. Known direct target genes of *ERG* in *TMPRSS2-ERG* fusion positive tissues include *MMP3*, *PLAU*, *LAMC2*, *KCNS3*, *PLA1A*, *C-MYC*, *GNMT*, *SARDH*, *CXCR4*, *ADAMTS1,TFF3*, *ERG*, *PLAT*, *MMP9*, *NDRG1*, *CUTL2*, *AR*, *KLK3 (PSA)*, *KLK2*, *SLC43A1*, *FKBP5*, *EZH2*, *ZBTB16*, *HPGD*, *ZEB1*, *SPINT1*, *IL1R2*, *PSMA*, and *OPN*. These and other up or down regulated genes in fusion positive cancers could facilitate PC progression. In addition, both *PTEN* loss and *AKT* overexpression facilitate the development of invasive carcinoma in *TMPRSS2-ERG* positive prostate tissues.

The Role of *TMPRSS2-ERG* Fusion Genes in the Clinical Prognosis of Prostate Cancer Patients

Since the *TMPRSS2-ERG* fusion was identified in 2005, there have been conflicting reports regarding the effects of the fusion on PC development, progression, aggressiveness, and clinical outcome. Several groups found no association between *TMPRSS2-ERG* fusion positive cancers and stage, grade, Gleason score, PSA-indicated recurrence, progression, prognosis, and/or disease aggressiveness [20-28]. *TMPRSS2-ERG* fusion PCs have additionally been linked to favorable prognoses. One group found *TMPRSS2-ERG* fusion positive status to be associated with favorable outcomes for patients undergoing radical prostatectomy [29]. Another group found fusion positive tumors to be associated with better survival and lower Gleason scores [30]. Hermans et al. [31] found a novel *TMPRSS2* transcript involved in fusion that starts at an alternative site, resulting in the inclusion of what they designated to be exon 0, which when included in the fusion is found to be associated with less aggressive behavior and therefore more favorable prognosis.

Fusion status has also been linked to poor outcomes and prognostic indicators. Fitzgerald et al. [28] found copy number increases involving the fusion gene are associated with decreased survival. A study by Gopalan et al. [32] also found copy number increase of fusions generated by deletion to be linked with aggressive disease. Another group identified a similar group of fusion positive tumors, identified as 2+Edel (signifying duplicated copies of fusions generated by deletion), to be associated with only a 25% survival rate after 8 years [33]. An increase in fusion copy number is also associated with poor prognosis [34]. As mentioned above, there are also reports that have indicated fusion formed via deletion, rather than translocation, to be associated with aggressive disease. One such report, by Mehra et al. [35] found that fusion positive metastatic sites were uniformly generated via deletion. Deletion generated *TMPRSS2-ERG* fusions have also been associated with higher tumor stage and lymph node involvement in PC patients [36].

TMPRSS2-ERG fusions have also been associated with biochemical or PSA indicated recurrence [37-39]. One study found 58.4% of fusion positive, versus 8.1% of fusion negative, patients to have recurrence within 5 years of treatment [38]. Another found that those with fusion positive tumors had a 5-year recurrence rate of 79.5% compared to 37.5% for fusion negative patients [40]. Barwick et al. [37] also found *TMPRSS2-ERG* fusion positive cancers to be statistically associated with biochemical recurrence. Rostad et al. [41] found *TMPRSS2-ERG* fusions to be associated with high levels of PSA, advanced stage, and high Gleason scores. Another group also found a link between *TMPRSS2-ERG* positive tumors and high Gleason scores [42]. In addition, cancers with *TMPRSS2-ERG* fusions have been associated with high grade tumors [43], and are more prone to metastasis [44]. Another study showed that there is a link between *TMPRSS2-ERG* fusion positive tumors and prostate specific death [45].

Different *TMPRSS2-ERG* transcript variants have also been linked to poor prognostic indicators. Wang et al. [13] found a variably expressed 72 base pair region, that, when present, was found to increase proliferation, invasiveness, and mortality. This group also found T2-E4 fusion to be associated with aggressive disease [13]. It has also been found that having more full length *ERG* than truncated *ERG*, lacking the *Ets* domain, is associated with poor differentiation, higher Gleason scores, and biochemical recurrence [46]. Additionally, T1-E2, T1-E3, and T2-E4 fusions all are associated with more aggressive disease, seminal vesicle invasion, and early PSA recurrence [16].

The tumor suppressor *PTEN* (phosphatase and tensin homolog) is a critical regulator of growth factors and inhibitor of PI3K. Loss of *PTEN* is frequently observed in prostate cancer, resulting in the deregulation of cell survival, growth, and proliferation. Previous studies have found that *PTEN* is lost or mutated in 30-80% of primary prostate cancer, and 50% of prostate cancer bone metastases. Concomitant *PTEN* loss and *TMPRSS2-ERG* fusion are associated with poor outcome. Carver et al. [47] found that *PTEN* loss combined with *ERG* rearrangement are statistically independent predictors of biochemical recurrence. Another study showed that cancers with no rearranged *ERG* and normal *PTEN* expression were statistically associated with good prognoses [48]. A study by Mosquera et al. [49] also shows the *TMPRSS2-ERG* fusion to be associated with certain morphological features. These features, including cribriform growth, blue tinged mucin, macronucleoli, and collagenous micronodules, are characteristic of PC.

It seems that the majority of findings indicate that the presence of *TMPRSS2-ERG* fusion gene expression in PC patients is associated with poor clinical prognosis. Evidence suggests that different subclasses of the *ERG* fusion transcripts may yield different clinical outcomes, such as 2+Edel, T2-E4, and +72bp transcripts being associated with worse clinical outcome. If the latter is true, then future prognostic studies may reconsider grouping all *ERG* fusion transcript variants into one prognostic parameter when analyzing PC cohort samples, as this may skew statistical results. A better understanding of the biological mechanisms employed by the *ERG* fusion proteins in PC progression may help to divide large cohorts into more effective subsets for prognostic analyses. With emerging evidence suggesting the involvement of the *ERG* fusion proteins in metastatic and advanced PC, it seems likely that the presence of the fusion proteins in PC contributes to a poor clinical prognosis. However, the fact that several large cohort studies have revealed opposing results regarding the prognosis value of *TMPRSS2-ERG* fusion gene expression in PC warrants further investigation into this matter.

Role of *TMPRSS2-ERG* gene fusions in prostate cancer progression

It has been known since 2005 that gene fusions are found in the majority of PCs [2]. The role that these fusions play in the development and progression of PC is much less understood. Since the discovery of the gene fusions, several groups have identified a collection of pathways and effects associated with the overexpression of *ERG* found in *TMPRSS2-ERG* fusion positive PCs. Recent studies have revealed several of the protein-protein interactions and target genes of *ERG* fusions, lending insight into *ERG* transcriptional regulation. Many of these studies have elucidated a key involvement of *ERG* fusions with the androgen receptor (AR) in hormone naïve, androgen sensitive prostate cancers. Despite castration resistant prostate cancer (CRPC) displaying partial independence from AR regulation, the expression of *TMPRSS2-ERG* fusion proteins in castration resistant prostate cancer (hormone refractory PC) has been shown to persist.

ERG overexpression in mouse models induces prostate cancer progression

Tomlins et al. [50] was one of the first groups to study the biological effects of *TMPRSS2-ERG* fusions in PC. To this end, transgenic mice

expressing a truncated version of *ERG* (exon 2 thru the native stop codon) under the control of a probasin promoter were generated and identified as *ARR2Pb-ERG* mice. In these *ARR2Pb-ERG* mice, 3/8 developed murine prostatic intraepithelial neoplasia (mPIN) by 12-14 weeks of age. Klezovitch et al. [51] found that mice expressing high levels of *ERG* under probasin promoter control developed mPIN by five to six months of age, and mice expressing comparatively lower, but still higher than normal, levels of *ERG* developed mPIN in 10-12 months. In *ERG* overexpressing murine prostate, there was disruption of the basal cell layer when compared to benign glands, an indicator of early stages of PC development. These observations were further supported by tissue recombination studies with *ERG* overexpressing prostate epithelial cells implanted in renal capsule showing disruption of basal epithelial cells [52]. Subsequently, further studies showed that *ERG* overexpression in mouse prostate does not result in high grade PIN lesions but rather subtle changes in mice prostate epithelial cells [47,53]. These differences in observed phenotypes were attributed to the different genetic backgrounds of the mice used in these studies and different isoforms of *ERG* gene used in overexpression studies. Carver et al. [47] found that *ERG* levels in *PTEN* knockout mice to be significantly higher than in controls. This group additionally generated *PTEN* haploinsufficient mice with *ERG* expression driven by the probasin promoter. These mice developed HGPIN at approximately two months of age and multifocal adenocarcinoma by six months of age, whereas control *PTEN* heterozygous mice developed HGPIN at approximately eight months of age and did not develop adenocarcinoma [47]. Comparable results were found by King et al. [53] in a study in which *TMPRSS2-ERG* mice were crossed with *PTEN* haploinsufficient mice. All of these mice (8/8) showed PIN development by six months of age, compared with only 1/8 littermate controls. These studies demonstrate that *ERG* cooperates with *PTEN* loss leading to the development of adenocarcinoma. This observation was also supported by tissue recombinant studies, where *ERG* overexpression in combination with either *PTEN* knockdown or expression of constitutively activated Akt resulted in development of adenocarcinoma [52]. The studies considered so far have all examined *ERG* overexpression alone or in combination with other genetic alterations. Alternatively, Sun et al. [54] and Wang et al. [13] investigated the biological effects of *ERG* knockdown using *TMPRSS2-ERG* fusion positive VCaP cells in xenograft model systems. When *ERG* expression was knocked down using siRNA in VCaP cells in a xenograft model, only 2/9 of the *ERG* siRNA SCID mice developed tumors by day 42, compared to 5/5 SCID mice in the control group [54]. Wang et al. [13] similarly investigated *ERG* expression knockdown in a mouse orthotropic model using stably transfected shRNA (short hairpin RNA) VCaP cell lines. After four weeks the scrambled shRNA control animals had a luciferase signal and tumor weight 4-fold that of the *ERG* shRNA animals. Together, these xenograft studies show that *ERG* expression in VCaP cells promotes tumor growth.

The findings from these mouse studies indicate that *TMPRSS2-ERG* fusion is not, on its own, sufficient to induce the development of invasive carcinoma. However, these fusions can cause the formation of PIN lesions. These studies also indicate that *PTEN* loss and *ERG* overexpression cooperate in the formation of PIN and invasive carcinoma, indicating that *PTEN* loss could function as a "second hit" in *TMPRSS2-ERG* fusion positive PC. *TMPRSS2-ERG* fusions seem to further associate with the PI3K pathway through cooperation with active Akt, the combination resulting in the development of invasive carcinoma.

ERG overexpression in prostate cell culture models increases cell invasiveness

In addition to animal models, cell culture-based studies have also been used to elucidate the biological role of *ERG*. Knockdown of *ERG* by siRNA in VCaP cells significantly inhibits their invasiveness [13,50,54]. Blocking urokinase plasminogen activator (uPA) and plasminogen activator pathways, which have been associated with *ERG* overexpression, as well as siRNA knockdown of uPA, were also shown to significantly inhibit cellular invasiveness in RWPE, VCaP, and BPH-1 cells [50,51].

Conversely, *ERG* overexpression has also been found to have many effects in cell culture studies. *ERG* overexpression in RWPE and PrEC cells was found to significantly increase their invasion [50]. *ERG* overexpression was also shown to increase invasiveness in PNT1a cells [13]. Additionally, *ERG* overexpression in BPH-1 cells was shown to increase proliferation rates and invasiveness [51]. Proliferation rates were also shown to increase in *ERG* overexpressing PrEC cells [13]. The chemokine receptor CXCR4 increases cellular invasiveness in VCaP cells; gene expression of CXCR4 is directly upregulated in the presence of R1881 treatment via the *ERG* fusion protein binding to the CXCR4 promoter [55]. In an *in vitro* chemoinvasion assay, VCaP cells invaded through a matrigel chamber at a significantly higher rate in the presence of the CXCR4 ligand, CXCL12, as a chemoattractant. The chemoinvasion rate was enhanced further when VCaP cells were treated with synthetic androgen R1881, suggesting that AR activation and subsequent *ERG* fusion upregulation induced increased CXCR4 cell surface expression, leading to increased chemoinvasion [55]. This suggests another mechanism of enhanced cellular invasion mediated by the *ERG* fusion proteins. *ERG* overexpression in either BPH-1 or PNTla cells enhances cellular migration [13,47]. Interestingly, co-overexpression of multiple *ERG* transcript variants increases cell proliferation rates, suggesting a potential synergism between co-expressed *ERG* variants [13]. These cell culture studies indicate that *ERG* overexpression mediated by *TMPRSS2-ERG* fusion can result in increased invasiveness, cell proliferation, and cellular migration. These effects are due to the aberrant regulation of downstream target genes of *ERG*, which in turn are able to alter normal cellular activity.

ERG mediates transcriptional regulation at specific gene loci via direct protein-protein and DNA binding interactions

In vitro studies on *ERG* protein structure and interactions using co-immunoprecipitation (Co-IP) revealed that the *ETS* domain is involved in DNA binding and protein interactions. Studies showed that wild-type *ERG* proteins physically interacted through their *ETS* domain via heterodimerization with other *ETS* transcription factor family members such as Fli-1, *ETS*-2, Er81, and Pu-1 [3,5]. Different *ERG* isoforms are also involved in the formation of a ternary complex with AP1 family members Fos and Jun, forming an *ERG*/Fos/Jun complex. In the same study, *ERG* proteins were shown to physically interact via homodimerization and homo-iso-dimerization with the same isoform or with other *ERG* isoforms [3]. Another study verified that these wild-type *ERG* protein-protein interactions influenced the regulation and expression of the MMP1 and MMP3 gene promoter regions. *ERG* interacted with Fos and Jun in order to activate MMP1 promoter activity, and in contrast, *ERG* inhibited *ETS*-2-induced activation of MMP3 promoter activity [56]. As shown by Co-IP followed by mass spectrometry analysis or western blot analysis in VCaP cells and *ERG* fusion positive human prostate cancer tissues, *ERG* physically interacts with PARP1 and DNA-PKcs in a DNA independent manner and with

Ku70 and Ku80 in a DNA dependent manner. *ERG* interacts with all four proteins through its C-terminal region, and *ERG* interacts with DNA-PKcs specifically through the *ERG-ETS* domain amino acid Y373. These protein interactions mediate DNA double stranded breaks and transcriptional regulation. In this same study, PARP1, DNA-PKcs, Ku70, and Ku80 were shown via chromatin immunoprecipitation assay to bind to *ERG* target genes, and PARP1 and DNA-PKcs were required for *ERG* transcriptional activation of the gene PLA1 [57]. These results suggest that *ERG* can regulate gene transcriptional activity in a protein interaction dependent manner, which is most likely coupled with DNA binding transcriptional regulation.

ERG is associated with the aberrant expression of many genes. Several of these genes have been identified through chromatin immunoprecipitation (ChIP) assays to be direct binding targets of *ERG*. Tomlins et al. [50] used such an assay to show that *ERG* can directly bind the proximal promoter of Urokinase Plasminogen Activator (*PLAU)* and *MMP3*. ChIP has also identified the recruitment of *ERG* to the *C-MYC* promoter upstream *Ets* element [54]. This study demonstrated that *ERG* was recruited to the *PSA* enhancer and to the *prostein* promoter upstream *Ets* element, showing direct interaction of *ERG* with *PSA* expression [54]. Additionally, *ERG* binds the *GNMT* promoter and the *SARDH* promoter [58]. A ChIP assay also found that *ERG* directly binds both *CXCR4* and *ADAMTS1* promoter regions, and the expression of these two genes is upregulated in the presence of *ERG* overexpression [47]. Additionally, *ERG* binds directly to the CXCR4 gene promoter region in response to R1881 treatment [55]. In a study by Tomlins et al. [50] ChIP identified *LAMC2*, *KNCS3*, and *PLA1A* as direct *ERG* targets. *ERG* was shown to be recruited to many gene regions including, PLAU, PLAT, MMP9, NDRG1, CUTL2, AR, KLK3 (PSA), KLK2, SLC45A1, FKBP5, EZH2, and ZBTB16 [59]. *ERG* also cooperates with AR during the recruitment of *ERG* to the TFF3 gene locus [60]. One study identified *ERG* as a modulator of prostaglandin signaling and showed that *ERG* directly bound to the core gene promoter region of the HPGD gene. In this study, *ERG* siRNA in VCaP cells resulted in an increase in HPGD expression, suggesting that *ERG* negatively regulates expression of the HPGD promoter [61]. *ERG* also promotes epithelial to mesenchymal transition, and *ERG* directly binds the ZEB1, SPINT1, and IL1R2 gene regions [62]. A study by Flajollet et al. [63] revealed that *ERG* directly bound to an *ETS* binding sequence in the promoter of the osteopontin gene (OPN), which resulted in an increase in OPN gene expression. In another study, *ERG* was shown to negatively regulate the expression of the prostate specific membrane antigen (PSMA) by direct binding to the PSMA gene locus [64]. One recent study revealed an important novel finding that wild-type *ERG* gene expression is under the control of the *TMPRSS2-ERG* fusion protein. This was verified by ChIP assay showing that *ERG* bound directly to the wild-type *ERG* locus in VCaP fusion positive prostate cancers, but not in LNCaP fusion negative cancers [65]. This finding is important as it reveals a feed-forward mechanism of *ERG* overexpression in *TMPRSS2-ERG* fusion positive PC cells, further suggesting an important role of *ERG* overexpression in the progression of PC. These ChIP assays demonstrate that many genes are direct targets of *ERG*, providing a potential explanation for their aberrant regulation.

ERG and AR co-mediate transcriptional regulation of the *ERG* and AR gene loci, as well as their target genes in prostate cancer

The role of AR in *TMPRSS2-ERG* fusion positive PC cells has also been evaluated using cell culture studies. In VCaP cells, *ERG* physically interacts with AR, as verified by Co-IP. *In vitro* studies revealed that the *ERG*-AR protein-protein interaction was mediated through the *ERG-ETS* domain in a DNA independent manner. This same study revealed that *ERG* transcriptional regulation often occurred concomitantly with AR. ChIP assays demonstrate that *ERG* and AR co-occupy many androgen regulated genes, such as KLK3 (PSA) and AR. In this study, *ERG* had an inhibitory effect on AR mRNA and protein expression, as well as an inhibitory effect on the mRNA expression of several AR regulated genes, some of which were co-occupied by *ERG* and AR. *ERG* mediates its inhibitory effects via direct DNA binding and transcriptional repression of AR target genes and by protein-protein interactions with AR [59]. Another study showed that *ERG* regulated expression of TFF3 (an androgen regulated gene) via direct binding to the TFF3 gene region in hormone-naïve PC and in CRPC [60]. Interestingly, *ERG* suppressed TFF3 expression in hormone naïve prostate cancer, and induced TFF3 expression in CRPC.

It has been shown that AR positively regulates *ERG* expression and possibly *ERG* target gene expression. As mentioned previously, a study by Cai et al. [55] showed that the synthetic androgen R1881 upregulates *ERG* and CXCR4 expression in fusion positive VCaP cells. In addition, R1881 activation of *TMPRSS2-ERG* fusions functionally activates CXCR4 expression in VCaP cells [55]. These data suggest that increased CXCR4 levels following R1881 treatment are indirectly attributed to AR activation via the R1881/AR induced upregulation of *ERG*. Consistent with these results, treatment of VCaP cells with R1881 increased *TMPRSS2-ERG* expression in several other independent studies [2,64,66,67]. However, *TMPRSS2-ERG* expression was not increased after R1881 treatment in AR negative, fusion positive NCI-H660 cells [66]. As mentioned previously, treatment of androgen sensitive VCaP cells with R1881 increased both *TMPRSS2-ERG* and wild-type *ERG* transcript expression. However, only the *TMPRSS2-ERG* transcript was directly increased by AR signaling, whereas wild-type *ERG* transcript was increased indirectly via the *TMPRSS2-ERG* fusion binding directly to the wild-type *ERG* locus [65]. Interestingly, AR signaling is implicated in a causal role in the formation of the *TMPRSS2-ERG* gene fusion rearrangements, further suggesting a strong involvement of AR with *TMPRSS2-ERG* gene fusions [68-71].

Expression of the *TMPRSS2-ERG* gene fusion persists in patients with Castration Resistant Prostate Cancer

TMPRSS2 gene expression is normally under the control of AR, so whether *TMPRSS2-ERG* fusions play a role in CRPC (hormone-refractory prostate cancer) has remained a controversial topic that is currently being investigated. A study by Hermans et al. [19] showed that although *TMPRSS2-ERG* gene fusions were present in four androgen independent prostate tumor xenografts, *ERG* fusion mRNA transcript was not detected in any of these fusion positive samples. Three of the four androgen independent prostate tumor xenografts also showed little to no expression of AR mRNA transcript, with the fourth showing only a low level of AR transcript expression. Another study showed that two AR negative, hormone-refractory xenografts LuCaP49 and LuCaP93 [72,73] were also *TMPRSS2-ERG* fusion positive, but did not express the *ERG* fusion transcript [29]. Thus, it seems that with the exception of the NCI-H660 cell line, AR negative PC cells harboring the *TMPRSS2-ERG* gene fusion generally do not express *ERG* fusion transcripts.

Despite these findings, several other studies have consistently shown that *TMPRSS2-ERG* fusion gene is present and expressed in AR positive CRPC patient tumors and xenografts [74]. Cai et al. [74] showed that *TMPRSS2-ERG* gene fusion mRNA expression was present in VCaP xenografts from castrated mice as well as in 11 of 29 (38%) of CRPC patient samples. These same fusion positive CRPC patient samples also showed increased AR mRNA expression levels relative to androgen dependent prostate cancer samples. This suggests that AR may continue to play a role in *TMPRSS2-ERG* gene expression even in a CRPC state. Additionally, *ERG* mRNA expression in VCaP mouse xenografts was decreased four days after mice underwent castration surgery. However, after approximately six weeks mice experienced tumor relapse, and *ERG* mRNA expression increased above levels seen prior to the castration surgery. These same xenograft mice showed progressively increasing AR mRNA and protein levels throughout the six week relapse period, consistent with the AR overexpression seen in the CRPC patient samples [74]. Relapse is a common occurrence among prostate cancer patients who undergo androgen deprivation therapies (ADT) in the clinic, so these findings seem to closely mimic the CRPC clinical setting. At the time of relapse, this same xenograft study found that there was an upregulation in the mRNA expression of several enzymes present in the DHT biosynthetic pathway [74]. Additionally, several studies have also shown that prostate cancer cells can synthesize androgens *de novo* [75,76]. Therefore, upregulation of DHT biosynthetic enzymes and *de novo* androgen synthesis by prostate cancer cells suggests a possible mechanism in which AR signaling and *TMPRSS2-ERG* expression may be reactivated in a CRPC state, possibly contributing to ADT resistance. These findings suggest a role for *TMPRSS2-ERG* in the progression of CRPC, although the mechanism of how CRPC cells bypass ADT and continue the AR induced expression of *TMPRSS2-ERG* remains to be fully characterized. It has been suggested that *TMPRSS2-ERG* expression may eventually become androgen independent in CRPC, but no data have yet confirmed this hypothesis; further studies will be needed in order to investigate this possibility [77].

Mehra et al. [35] found that *TMPRSS2-ERG* mRNA expression was present in 10 of 27 (37%) of hormone-refractory metastatic prostate cancer patient samples. Of these 10 patients, 100% of metastatic sites contained a *TMPRSS2-ERG* fusion formed through interstitial deletion (Edel). In accordance with these findings, all metastatic tumor sites in one individual harbored the same fusion subtype, and this same fusion subtype was also found in the primary tumor site of the prostate. This suggests a mechanism of clonal selection and clonal expansion of the *TMPRSS2-ERG* fusions in metastatic fusion-positive CRPC. Attard et al. [67] found similar results, with 9 of 15 (60%) CRPC tumor samples expressing the *TMPRSS2-ERG* transcript, and circulating tumor cells (CTCs) collected from 11 CRPC patients showing the same *ERG* gene status as prostate tumor tissues from the same individual.

Many studies have revealed the presence of *TMPRSS2-ERG* transcript in CRPC; Rickman et al. [60] revealed a unique biological role of the *TMPRSS2-ERG* fusions in CRPC. In this study, 14 of 19 (73%) *TMPRSS2-ERG* fusion positive CRPC samples were found to express the *ERG* fusion transcript. Importantly, this study found that *ERG* fusions induced expression of TFF3 in CRPC, but inhibited TFF3 expression in hormone-naïve PC samples. Additionally, *ERG* fusion regulation of TFF3 was dependent on AR signaling, and overexpression of TFF3 in fusion positive CRPC resulted in an increase in cellular invasiveness. This study revealed a novel functional role of *TMPRSS2-ERG* fusion proteins in the progression of CRPC, and that the function of *ERG* fusions can change depending on the selective pressures and progressive state of the disease.

Concluding Remarks

Overall, the studies discussed here support a model (Figure 1) in which circulating androgens in PC patients activate *TMPRSS2-ERG* fusions, with the resulting *ERG* protein regulating genes whose expression and function facilitate PC progression. As mentioned above, this can increase invasiveness, cellular motility, and disease aggressiveness. In addition, it has been shown to cause the upregulation of oncogenes, as well as the downregulation of prostate differentiation genes. This, as well as the other evidence outlined above, indicates the importance of *TMPRSS2-ERG* fusions in PC. Considering the prevalence of *TMPRSS2-ERG* fusion in PC, and the large number of men who develop PC each year, the further study of these fusions is vital to the understanding of PC biology. Once better elucidated, the associations between this fusion and prognostic indicators and disease aggressiveness could give clinicians a more informed way of classifying PC for prognostic and treatment purposes. The furthered understanding of *ERG*'s biological roles in PC will augment this and potentially provide novel target pathways for future therapies, potentially through inhibition of *ERG* itself. Continued research of gene fusions, in particular *TMPRSS2-ERG*, is needed to better understand their biological roles and relationship to PC development and progression.

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